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A RECOMBINANT PLASMID AND A METHOD OF CONTROLLING THE EFFECTS OF *YERSINIA PESTIS*

Licensing Rights

The United States government may have licensing rights to this application in accordance with U.S. Public Health Service Grant AI 19353.

Technical Field

The present invention is concerned with bubonic plague caused by *Yersinia Pestis* and vaccines for treating same.

Background Art

Experimental plague in mice, caused by *Yersinia pestis*, is mediated by two distinct types of virulence factors. Members of the first category serve as whole animal or tissue invasins by promoting dissemination of the organisms into visceral organs following infection by peripheral routes of injection (e.g. intraperitoneal or subcutaneous). Mutants lacking one or more tissue invasins can exhibit significant reduction in virulent (50% lethal dose > 10^2 to 10^7 bacteria) by peripheral administration but retain essentially full lethality (50% lethal dose ca. 10^2 bacteria) upon intravenous injection (4). Examples of this group (6, 16, 48) include the outer membrane (46) plasminogen activator (1) mediated by a ca. 10 kb pesticin or Pst plasmid (12, 41) and a series of iron repressible outer

membrane peptides (10, 40) encoded by a delectable ca. 100 kb chromosomal segment (11, 22).

Examples of the second category function to promote lethality following infection by the intravenous route, known to facilitate immediate transport of the bacteria to favored niches within visceral organs (4). Mutational loss of these lethal factors causes qualitative (intravenous 50% lethal dose > 10^7 bacteria) decreases in virulence. Included in this group are certain ca. 70 kb low calcium response or Lcr plasmid encoded proteins: V antigen (9, 27), others termed Yops (18, 19, 33, 47): YopE (23, 35, 43, 44, 47), YopH (3, 34, 44), and probably YpkA (13), as well as chromosomally encoded antigen 4 or PH 6 antigen (20) and possibly the murine exotoxin encoded by the ca. 100 kb Tox plasmid (32.). Considerable effort has been spent in study of the regulation, processing, and delivery of these proteins to host cells (2, 15, 26, 28, 30, 31, 35).

The effectiveness of the immune response directed against members of the second category of virulence factors has only been reported for V antigen. Some (17, 24, 27, 39, 49, 50) but not all (5) antibodies directed against this 37 kDa exported (8, 17, 43, 44) protein provided significant passive protection against experimental plague in mice. This effect was associated with release of a potent immunosuppressive block preventing both synthesis of cytokines (27) and formation of protective granulomas (50).

Plague vaccines have been identified in U.S. Patent No. 3,137,629. The patent describes a process

for producing killed plague vaccines which immunizes mice and guinea pigs by growing *Pasteurella pestis*, killing the strain through mechanical action and solubilizing the extract in strong alkaline solution, and then preparing parental vaccine by reducing the pH value of the soluble *P. pestis* antigenic solution to a neutral pH.

It is an object of the present invention to prepare a plasmid by recombinant techniques.

It is another object of the present invention to prepare an antigen encoded by the recombinant plasmid.

It is a further object of the present invention to control the effect *Y. pestis* has on mammals by utilizing a vaccine to *Y. pestis* constituting the antigen noted above.

Summary Of The Invention

The present invention is concerned with a plasmid prepared by recombinant techniques having the construct shown in Figure 1.

Also described is a protein encoded by the plasmid shown in Figure 1, capable of inducing a protective antibody response.

The invention is further concerned with a method of controlling the effects of *Y. pestis* in mammals comprising the step of:

a) providing a vaccine comprised of the protein encoded by the construct of Figure 1; and

b) treating a mammal in need thereof with an effective anti-*Y. pestis* amount of the vaccine.

Brief Description Of The Drawings

FIGURE 1A is a scheme of construction of a recombinant plasmid formed by joining the DNA encoding the signal sequence and IgG binding domains of staphylococcal protein A and that of all but the 201 N-terminal base pairs of *Y. pestis* V antigen; antibody to which is capable of effecting immunological treatment against *Y. pestis*;

FIGURE 1B cites a restriction endonuclease attack on the V antigen;

FIGURE 2 is a silver-stained extended SDS gel of whole cells of E. coli BL 21 containing the vector plasmid pKK223-3 (lane 1) or recombinant plasmid pKVE14 (lane 2);

FIGURE 3 are immunoblots prepared with rabbit polyclonal anti V antigen (A) mouse monoclonal anti V antigen 15A4.8 (B) and mouse monoclonal anti V antigen 3A4.1 (C) directed against whole cells of E. coli containing the vector plasmid pKK223-3 (lane 1) or recombinant plasmid pKE14 (lane 2);

FIGURE 4 are immunoblots prepared with rabbit anti-native V antigen purified from *Y. pestis* KIM (A), anti-recombinant V antigen (B), anti-protein A-V antigen fusion protein (C), and anti-Protein A (D) directed

against Ca^{2+}-starved whole Lcr^- cells of *Y. pestis* KIM (lane 1), Lcr^+ cells of *Y. pestis* KIM (lane 2), Lcr^- cells of *Y. pseudotuberculosis* PB1 (lane 3), Lcr^+ cells of *Y. pseudotuberculosis* PB1 (lane 4), Lcr^- cells of *Y. enterocolitica* WA (lane 5), and Lcr^+ cells of *Y. enterocolitica* WA (lane 6);

FIGURE 5 is a chart of antiserum for passive immunization against native V antigen, recombinant V antigen, recombinant protent A-C antigen fusion and recombinant protein A;

FIGURE 6 are immunoblots prepared with rabbit anti-native V antigen (A) or mouse monoclonal 17A5.1 anti V antigen (B) directed against truncated protein A (PA) (lane 1), protein A-V antigen fusion peptide (PAV) (lane 2), PAV partially hydrolyzed by formic acid (lane 3), PAV partially hydrolyzed formic acid and passed through the IgG Sepharose 6FF column (lane 4), whole Lcr^+ cells of *Yersinia pestis* KIM (lane 5), and whole LCR^- cells of *Y. pestis* KIM (lane 6); A-V_d, V_o, V_d, and A indicate the positions of PAV, native V antigen (37 Kda), truncated V antigen (29.5 kDa), and truncated Protein A, respectively. Human γ-globulin was used to block nonspecific reactions of monoclonal antibodies against IgG-binding domains of Protein A (26); and

FIGURE 7 are immunoblots prepared with rabbit anti-protein A-V antigen fusion peptide (A) and anti-truncated protein A (B) directed against whole cells of *Escherichia coli* containing the vector plasmid pKK223-3 (lane 1) or recombinant plasmid pKE14 (lane 2); Also shown are reactions against disrupted and centrifuged whole cells of *E. coli* (pKE14) (lane 3) and further

fractionation of V antigen by chromatography on phenyl-Sepharose CL-4B (lane 4), DEAE (diethylaminothyl) cellulose (lane 5), Sephacryl S-300SF (lane 6), calcium hydroxylapatite (lane 7), and a second passage on DEAE cellulose (lane 8).

Description of the Best Mode

The medically significant yersiniae (*Yersinia pestis, Y. pseudotuberculosis,* and *Y. enterocolitica*) are known to share a ca. 70 kb low calcium response (Lcr) plasmid that mediates restriction of vegetative growth at 37°C in Ca^{2+}-deficient media while promoting selective synthesis of virulence factors including V antigen. The latter, encoded by *lcrV* on the Lcr plasmid, is established as a 37 kDa protective antigen capable of undergoing possible autoproteolytic hydrolysis. In this study, *lcrV* of *Y. pestis* was cloned under control of the strong *tac* promoter into protease-deficient *Escherichia coli* BL21. The resulting recombinant V antigen, like native V antigen, underwent degradation during purification yielding major peptides of ca. 36,35,34 and 32 to 29 kDa. Rabbit γ-globulin raised against this mixture of cleavage products provided partial but significant protection against 10 minimal lethal doses (MLD) of the three species. To stabilize V antigen and facilitate its purification, plasmid pPAV13 was constructed so as to encode a fusion of *lcrV* and the structural gene for staphylococcal protein A (e.g. all but the first 67 N-terminal amino acids of V antigen and the signal sequence plus IgG binding domains but not cell-wall associated region of protein A). The resulting protein A-V antigen fusion peptide (PAV) could be purified to homogeneity in one step IgG affinity

chromatography and was found to be stable thereafter. Rabbit polyclonal γ-globulin directed against PAV provided substantial passive immunity against 10 MLD of *Y. pestis* and *Y. pseudotuberculosis* but was ineffective against *Y. enterocolitica*.

The vaccine as described herein is generally applied by parental administration to mammals in need thereof.

The vaccine of the present invention is generally administered in the form of pharmaceutical compositions comprising a pharmaceutically acceptable vehicle or diluent. Such compositions are generally formulated in a conventional manner utilizing liquid vehicles or diluents as appropriate to the mode of desired administration: for parenteral administration (e.g. intramuscular, intravenous, intradermal), in the form of injectable solutions or suspensions, and the like. For use as a vaccine in a mammal, including man, it is given in an amount of about 0.5-100 mg/kg.

The vaccine described herein is used in conjunction with normal pharmaceutical excipients to facilitate storage and use.

The present invention is further illustrated by the following examples. These examples are provided to aid in understanding of the invention and not to be construed as a limitation thereof.

Materials and Methods

Bacteria. *Escherichia coli* K-12 XL1-Blue {recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F' proAB $lacI^qZ\Delta M15Tn10$ (tet^r)]} (Stratagene, La Jolla, Calif.) was used as a host for genetic engineering manipulations. Protease-deficient *E. coli* BL21 {F^- ompT lon r_B- m_B-} (Novagen, Madison, Wisc.) was used for expression of cloned genes (17). Mice passively immunized with the products of cloned genes were challenged with wild type cells of *Y. enterocolitica* WA (10) or *Y. pseudotuberculosis* PB1/+ (9). This purpose was accomplished with *Y. pestis* KIM by use of a nonpigmented mutant (20,59) known to lack a spontaneously deletable ca. 100 kb chromosomal fragment encoding functions of iron transport and storage (14,28); the isolate in question retained all other known chromosomally encoded virulence functions as well as the Tox, Lcr, and Pst plasmids (13,57). Nonpigmented mutants of this phenotypic background are virulent in mice by the intravenous (50% lethal dose ca. 10 bacteria, 61) but not by peripheral routes of infection (50% lethal dose $>10^7$ bacteria, 21).

Plasmids. Salient features of plasmids used in this study are shown in Table 1. The vector pKK223-2 containing the *tac* promoter (Pharmacia, Uppsala, Sweden) was used to express a portion of the *lcr*GVH-yopBD operon of *Y. pestis* 358 (22) as described below. The vector pRIT5 (Pharmacia) encoding the sequence of Protein A of *Staphylococcus aureus* was used for preparation of gene fusions. The recombinant plasmid pBVP5 containing the lcrGVH-YopBD operon of *Y. pseudotuberculosis* (38) served as the source of *lcrV* in preparing this construction.

Table 1

Characterization of Deletional Variants of HindIII Fragment From The *lcrGVH-yopBD* Operon of *Yersinia* Pseudotuberculosis 995.

Encoding Plasmid	Size Of Fragment	Operon	Designation Of V Antigen	Size of V Antigen (kDa)
pBVP5	~3,500	lcrgvh-ypoB	V_0	37.3
pBVP513D	2,184	lcrGVH	V_0	37.3
pBVP53D	1,484	$lcrGV_1$	V_1	31.5
pBVP514D	1,160	$lcrGV_2$	V_2	19.3
pBVP515D	878	$lcrGV_4$	V_3	8.5
pBVP58D	705	$lcrGV_4$	V_4	2.0
pBVP55D	546	$lcrG_1$	-	-

Molecular weights of truncated V antigens were calculated from the deletion terminus as determined by nucleotide sequencing; actual values may be slightly greater due to translational overruns into the vector polylinker region.

DNA Methods. The preparation of plasmid DNA, digestion with restriction enzymes, ligation, and transformation of *E. coli* were undertaken essentially as described by Maniatis et al (29). The 3.5 kb HindIII fragment of the Lcr plasmid of *Y. pestis* 358 (22,38) was introduced into expression vector pKK223-3. The resulting recombinant plasmid pKVE14 was then selected where the direction of transcription of the lcrGVH sequence corresponds to the direction of action of the tac promoter.

The schema used to construct pPAV13 containing a hybrid gene encoding a portion of Protein A of S.

aureus and *lcrV* of *Y. pseudotuberculosis* is shown in Figure 1A. The 1.5 kb EcoRV fragment of recombinant plasmid pBVP5 (38) was introduced into the vector pRIT5 encoding truncated Protein A (PA). The latter, either alone or fused with V antigen, maintained its signal sequence and most IgG-binding domains but lost the region mediating association with the bacterial cell surface (41,42)(Fig. 1B). PA does not contain cysteine and is thus unable to form disulfide bridges between itself and a hybrid domain (60). As a consequence of this fusion, *lcrV* lost 201 bp which thus deleted the first 67 amino acids comprising the N-terminal portion of V antigen. The resulting Protein A-V antigen fusion peptide (PAV) thus contained 305 N-terminal amino acids from Protein A and 259 C-terminal amino acids from V antigen (Figure 1B).

<u>Purification of Recombinant V Antigen</u>. Cells of *E. coli* BL21 (pKVE14) were grown in fermenters as described previously (5) in medium containing 3% Sheff-ield NZ Amine, Type A (a pancreatic hydrolysate of casein which contains mixed amino acids and peptides and is used to facilitate the growth of bacteria) (Kraft, Inc., Memphis, Tenn.), 0.5% NaCl, 1% lactose, and ampicillin (100 μg/ml) at 37°C and harvested by centrif-ugation (10,000 x g for 15 min) at an optical density (620 nm) of about 1.2. After disruption in a French pressure cell (SLM Instruments, Inc., Urbana, Ill.) and removal of insoluble matter by centrifugation (10,000 x g for 30 min), V antigen was subjected to purification by an established procedure (5). The method involved use of hydrophobic interaction chromatography with Phenyl-Sepharose CL-4B (Pharmacia), ion exchange chroma-tography with DEAE cellulose (Whatman Inc., Clifton,

J.J.), gel filtration chromatography with Sephacryl S-300SF (trademark of Pharmacia Biotechnology Group for acrylic resin for chromatograhic separation of proteins), and Bio-Gel HTP (trademark of Bio-Rad, Richmond, California, for calcium hydroxyapatite (chromatography)). The original procedure was supplemented by a second chromatographic separation of DEAE cellulose (linear gradient from 0. 0.35 M NaCl) in order to remove high molecular weight material peculiar to *E. coli*.

Preparation of PA and PAV. Cells of *E. coli* transformed with pPAV13 or PRIT5 were grown to late log phase at 37°C in Luria broth containing ampicillin (50μg/ml). Purification of these recombinant proteins was accomplished by affinity chromatography on IgG Sepharose 6FF (Pharmacia) according to directions supplied by the manufacturer. Briefly, the procedure involved harvesting the organisms by centrifugation (10,000 x *g* for 15 min) with resuspension at a ca. 10-fold increase in number in 0.01 M Tris.HCl, pH 8.0 (column buffer). Lysis was accomplished by addition of lysozyme (5 Mg/ml) and, after incubation for 1 h, further addition of Triton X-100 (trademark of Aahm & Haas Co. for a nonionic detergent comprised of octyl phenopypolyethoxy ethanol having an HLB: 13.5$_{j}$ (0.1%) whereupon incubation was continued for 3 to 4 h. After clarification by centrifugation (10,000 x *g* for 30 min), samples of 400 ml of the resulting enriched periplasm were passed through a column (10 x 100 mm) containing a 10 ml packed volume of affinity resin that selectively bound PA or PAV. After addition and elution of 10 void volumes of column buffer to remove contaminating matter, the recombinant proteins were eluted with 0.2 M acetic acid (ca. pH 3.4), immediately frozen, and the lyophi-

lized. Resulting purified PA and PAV were then used directly for qualitative analysis and immunization.

Acid Hydrolysis of PAV. Purified PAV was treated with 70% formic acid for 20 h at 30° C to cleave the four labile Asp-Pro peptide bonds within the Protein A domain (60) and the additional site located at the junction with V antigen (41) (Fig. 1B). After dialysis against column buffer, the partial hydrolysate was again passed through the IgG Sepharose 6FF column as described above. In this case, the V antigen moiety plus fragments of PA lacking IgG binding sites were immediately eluted whereas residual unhydrolyzed PAV remained bound to the affinity resin.

Antisera. The same lot of refined rabbit polyclonal anti-V antigen characterized previously (40) was used as a positive immunological control. Monoclonal antibodies directed against V antigen have been defined (3). These reagents consisted of two groups: monoclonals 3A4.1, 17A5.1, and 17A4.6 that reacted with nonconformational epitopes located within the last 50 amino acids comprising the C-terminal part of V antigen (amino acids 276 to 326) and monoclonal 15A4.8 that reacted with an internal nonconformational epitope located between amino acids 168 to 275 (37).

Rabbit polyclonal antisera was raised against PA and PAV with Freund's adjuvant as described previously (62). TiterMax™ adjuvant (Hunger's TiterMax #R-1, CytRx Corp., Norcross, Ga.) was used to immunize rabbits against recombinant V antigen plus its degradation products purified from *E. coli* BL21 (pKVE14). Antisera prepared against recombinant V antigen or fusion pro-

teins were not absorbed with material from Lcr bacteria although highly purified γ-globulin was isolated from these reagents as described previously (62). Antisera raised against V antigen purified from *Y. pestis* or *E. coli* BL21(pKVE14) is termed anti-native V antigen or anti-recombinant V antigen, respectively.

Immunoblotting. Alkaline phosphatase conjugated with anti-rabbit or anti-mouse IgG (Sigmal Chemical Co., St. Louis, Mo.) were used as secondary antibodies in immunoblotting by procedures essentially identical to those already defined (51,52). In order to prevent nonspecific reactions of antibodies with PA and PAV, the nitrocellulose filter was first blocked with 5% fetal calf serum as usual and then incubated overnight in a solution of 1% normal human γ-globulin (Calbiochem, San Diego, Calif.). Human γ-globulin (0.5%) was also added to solutions of primary and secondary antibodies (26). In addition, Fc-specific anti-mouse IgG (A-1418, Sigma) was used as a secondary antibody during immunoblotting of fusion proteins and their derivatives with monoclonal antibodies.

Passive Immunity. The ability of highly purified Υ-globulin obtained from unabsorbed rabbit polyclonal antisera raised against recombinant V antigen, PA, and PAV to provide passive immunity was assayed by defined methods (40,62). Briefly, this procedure involved intravenous injection of 10 minimum lethal doses (MLD) of *Y. pestis* (10^2 bacteria), *Y. Pseudotuberculosis* (10^2 bacteria), or *Y. enterocolitica* (10^3 bacteria) followed by intravenous administration of either 100 μg or 500 or 500 μg of purified γ-globulins on postinfection days 1, 3, and 5.

<u>Miscellaneous</u>. Peptides were located in sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels, prepared as defined previously (51,52), by silver staining (36). Soluble protein was determined by the method of Lowry et al. (27).

Results

<u>Degradation of Recombinant V Antigen</u>. Recombinant plasmid pKVE14 containing the *lcrGVH-yopBD* operon of *Y. pestis* under control of the *tac* promoter was transferred into protease-deficient *E. col* BL21. After growth in fermenters, the bacteria were disrupted and the resulting extract was used to prepare nearly homogenous recombinant V antigen using a method established for Ca^{2+}-starved cells of *Y. pestis* (5). An additional step involving a second separation with DEAE cellulose was necessary to eliminate major higher molecular weight proteins present in *E. coli* cytoplasm.

The initial specific activity of recombinant V antigen was almost 5-fold greater than that obtained from *Y. pestis* starved for Ca^{2+} (5). Nevertheless, significant loss of precipitin activity occurred during every step of purification (Table 2). This phenomenon, as judged by a silver-stained extended lane gel (Fig. 2), reflected gradual loss of the native 37 kDa form with emergence of ca. 36 kDa, 32 kDa, and possibly smaller peptides. Analysis by immunoblotting was undertaken to prove that these new peptides shared epitopes with and thus arose from native V antigen. Use of rabbit polyclonal anti-native V. antigen (Fig. 3A) or mouse monoclonal antibody 15A4.8, directed against a centrally located epitope (Fig. 3B), demonstrated

emergence of ca. 36, 35, and 34 kDa degradation products early during the course of purification with later appearance of a series of smaller fragments ranging from 32 to 29 kDa. The latter were not recognized by mouse monoclonal antibody 3A4.1 directed against an epitope located near the C-terminal end (Fig. 3C). These findings indicate that recombinant V antigen produced in protease-deficient *E. coli* BL21 undergoes evidence spontaneous degradation in a manner similar to that observed for native V. antigen expressed in *Y. pestis* (5). Furthermore, patterns observed upon immunoblotting with monoclonal antibodies indicate that the C-terminal portion of V is involved in this process.

Table 2

Purification Of Recombinant V Antigen From A Cell-Free Extract Of *Escherichia coli* BL21 (pKVE14)

Preparation	Vol. (ml)	Protein (mg/ml)	Total Protein (mg)	V Antigen (U/ml)	Total V-antigen (U)	Specific Activity	% Recovery
Crude Extract	200	26	5,200	280	56,000	11	(100)
Phenyl-Sepharose CL-4B	220	1.6	350	140	30,800	88	55
DEAE	40	1.5	60	170	6,800	113	12.1
Cellulose Sephacryl S300SF	24	0.7	17	140	3,360	200	6.0
Ca Hydroxylapatite	35	0.25	8.8	50	1,750	200	3.1
DEAE Cellulose	18	0.1	1.8	15	270	150	0.5

The unit of V antigen was defined as the reciprocal of the highest dilution capable of forming a visible precipitate against a standardized lot of rabbit polyclonal monospecific antiserum by diffusion in agar under conditions described previously (*Lawton et al*, 1963; *Brubaker et al.*, 1987).

Passive Immunity Mediated By Anti-Recombinant V Antigen. A portion of the purified lot of recombinant V antigen described above was used to immunize rabbits. Immunoblots of the resulting unabsorbed antisera (Fig. 4B) and control absorbed anti-native V antigen (Fig. 4A) versus Ca^{2+}-starved whole yersiniae were identical indicating that the reagent was monospecific. Both antisera were tested for ability to confer passive immunity against intravenous infection with yersiniae. As shown in Fig. 5, the control anti-native V antigen provided complete, partial, and insignificant protection against *y. pestis, Y. pseudotuberculosis,* and *Y. enterocolitica*, respectively. Anti-recombinant V antigen promoted a similar degree of passive immunity except that that directed against *Y. pestis* was not absolute.

Characterization of PA and PAV. Additional constructions encoding truncated staphylococcal Protein A either alone or fused with V antigen (Fig. 1) were found, after transformation into *E. coli* BL21, to promote significant synthesis of PA and PAV, respectively as judged by intensity of the specific immunoblots described below. PA and PAV were purified in one step with IgG Sepharose 6FF and then analyzed by immunoblotting. Anti-native V antigen reacted nonspecifically with PA (Fig. 6A, lane 1) and both specifically and nonspecifically with PAV (Fig. 6A, lane 2). Proof that the salient peptides shown in lanes 2, 3, and 4 of Fig. 6A reacted specifically rather than nonspecifically with anti-native V antigen was obtained by blocking PA with human γ-globulin and then immunoblotting with monoclonal anti V antigen. This process prevented visualization of PA (Fig. 6B, lane1) thus demonstrating that the remain-

ing detectable bands represent a specific interaction with an epitope of V antigen. Multiple bands appearing in samples of both PA and PAV (Fig. 6A, lanes 1, 2) reflect accumulation in the periplasm of *E. coli* BL21 of the synthesized PA domain in both native and degraded forms as described by others (16). To prove that the V antigen domain of the fusion protein was stable, a sample of purified PAV was hydrolyzed with 70% formic acid to cleave acid labile Asp-Pro sites defined in Fig. 1B, neutralized, and then applied to the affinity column. Essentially pure truncated V antigen (V_d) emerged immediately (Fig. 6, lane 4); the absence of multiple bands in this sample provides evidence for the stability of V antigen within PAV.

<u>Stability of PAV</u>. The number of total units of near-homogenous PAV recovered after chromatography on IgG Sepharose 6FF was always essentially identical to that present in the crude extract applied to the affinity column. No significant loss of purified PAV occurred during storage in 0.01 M Tris. HCl, pH 7.8 for 1 week at 4°C.

<u>Passive Immunity Mediated By Anti-PAV</u>. Preparations of homogenous γ-globulin were isolated from unabsorbed rabbit antisera raised against PA and PAV purified by affinity chromatography. Control anti-V antigen (Fig. 4A and 4B) and anti-PAV (Fig. ~~5~~4C), but not anti-PA (Fig. ~~5~~4D), reacted with V antigens of all three *Yersinia* sp. High molecular weight antigens (ca. 70 kDa) common to both Lcr^+ and Lcr^- yersiniae were recognized by both anti-PAV (Fig. 4C) and anti-PA (Fig. 4D). Anti-PAV (Fig. 7A) but not anti-PA (Fig. 7B) also identified the same degradation products of V antigen

that were detected by the antisera characterized previously (Fig. 3). These findings verify that anti-PAV contains antibodies directed against epitopes unique to V antigen and provide further evidence that its degradation occurs at the C-terminal end.

Anti-PA and anti-PAV were tested for ability to confer passive immunity against intravenous infection with yersiniae. As shown in Fig. 5, anti-PA failed to provide protection whereas anti-PAV was highly effective against *Y. pestis* and *Y. pseudotuberculosis* but not *Y. enterocolitica*.

Discussion

Since its discovery, V antigen has been ascribed a role as protective antigen in conferring immunity to plague (8). Experimental evidence supporting this assumption was initially limited to the findings that active immunization with V antigen-rich fractions or passive immunization with antisera raised against such fractions provided protection against disease (23). The possibility thus remained that one or more additional antigens contributed to the immune state described by early workers. This concern was minimized upon introduction of preparative methods that permitted recovery of nearly homogenous V antigen (5). Nevertheless, antisera raised against lots purified by use of these procedures often contained antibodies directed against highly antigenic contaminating proteins, especially Yops, present at trace levels in the final product used for immunization. Although these antibodies were readily removed by absorption with cross-reacting material, this process necessitated introduc-

tion of bacterial macromolecules including lipopolysaccharide that might stimulate nonspecific resistance to infection. To minimize this possibility, it became necessary to purify the γ-globulin from absorbed antisera (40,62).

Concerns that these precautions, undertaken to assure monospecificity of anti-V antigen, had inadvertently introduced uncontrolled variables were largely eliminated by use of γ-globulin purified from antisera raised against highly purified V antigen cloned in *E. coli*. However, this process was also unsatisfactory due to the occurrence of marked degradation throughout the course of purification. As a result, only a fraction of the final product consisted of the 37 kDa primary *lcrV* product. Although γ-globulin purified from unabsorbed antiserum raised against this mixture provided satisfactory passive immunity, yields of antigenic material were insufficient to permit widespread immunization. The observation that cloned V antigen expressed in the prototease-deficient background of *E. coli* BL21, like native V antigen purified from *Y. pestis*, underwent marked degradation during purification further suggests but does not prove that this process is autocatalytic.

Problems concerning specificity and degradation were resolved upon development of the fusion protein PAV that could be isolated at high yield as a homogenous stable protein in a single step. Antisera raised against PAV was somewhat more effective in providing protection against *Y. pestis*, and especially *Y. pseudotuberculosis* than was anti-recombinant V antigen. This finding emphasizes that passive immunity mediated by anti-V antigen does not require interaction

with N-terminal epitopes because the latter were absent in PAV. The independent observation that the N-terminal end of V antigen was poorly antigenic (37) is consistent with this conclusion.

Detailed information with respect to the drawing figures is as follows.

FIGURE 1. Scheme of construction of recombinant plasmid of pPAV13 encoding staphylococcal protein A-V antigen fusion protein (PAV) (A) and characterization of PAV (B). Sites of restriction endonuclease attack are designated; Ap and Cm are locations of markers of resistance for ampicillin and chloramphenicol, respectively. Lac designates the position of *lacZ* which provides selection of recombinant plasmids in the vector pBluescript SK+. The genes *lcrG, lcrV,* and *lcrH* comprise a portion of the *lcrGVH-yopBD* operon of *yersinia pseudotuberculosis* 995 (38) and the designation Protein A is the truncated Protein A gene of *Staphylococcus aureus.* The dark arrows in A represent the hybrid gene encoding PAV shown in B to consist of the signal sequence (S), IgG-binding domains (E to B), the defective domain C′ that has lost the ability to bind IgG, and truncated V antigen that has lost the first 67 amino acids of its N-terminal portion. Molecular weights in Kilodaltons are designated for each peptide arising after hydrolysis of the acid-labile Asp-Pro cleavage sites marked by arrowheads (60).

FIGURE 2. Silver-stained 12.5% extended sodium dodecyl sulfate-polyacrylamide electrophoresis gel of whole cells of *Escherichia coli* BL21 containing the vector plasmid pKK223-3 (lane 1) or recombinant

plasmid pKVE14 (lane 2). Whole cells of *E. coli* (pKVE14) were disrupted and centrifugal to prepare a cell-free extract (lane 3) that was fractionated by chromatography on phenyl-Sepharose CL-4B (lane 4), DEAE cellulose (lane 5), Sephacryl S-300SF (lane 6), calcium hydroxylapatite (lane 7), and a second passage on DEAE cellulose (lane 8). Note the presence of V antigen in lanes 2 through 8 as a major peptide of 37 kDa.

<u>FIGURE 3</u>. Immunoblots prepared with rabbit polyclonal anti-V antigen (A), mouse monoclonal anti-V antigen 15A4.8 (B), and mouse monoclonal anti-V antigen 3A4.1 (C) directed against whole cells of *Escherichia coli* containing the vector plasmid pKK223-3 (lane 1) or recombinant plasmid pKE14 (lane 2). Also shown are reactions against disrupted and centrifugal whole cells of *E. coli* (pKE14) (lane 3) and further fractionation of V antigen by chromatography on phenyl-Sepharose CL-4B (Lane 4), DEAE cellulose (lane 5), Sephacryl S-300SF (lane 6), calcium hydroxylapatite (lane 7) and a second passage on DEAE cellulose (lane 8).

<u>FIGURE 4</u>. Immunoblots prepared with rabbit anti-native V antigen purified from *Y. pestis* KIM (A), anti-recombinant V antigen (B), anti-protein A-V antigen fusion protein (C), and anti-Protein A (D) directed against Ca^{2+}-starved whole Lcr^- cells of *Y. pestis* KIM (lane 1), Lcr^+ cells of *Y. pestis* KIM (lane 2), Lcr^- cells of *Y. pseudotuberculosis* PB1 (lane 3), Lcr^+ cells of *Y. pseudotuberculosis* PB1 (lane 4), Lcr^- cells of *Y. enterocolitica* WA (lane 5), and Lcr^+ cells of *Y. enterocolitica* WA (lane 6).

FIGURE 5. Ability of 0.033 M potassium phosphate buffer, pH 7.0 (None) or control antiserum raised against native V antigen and experimental antisera raised against recombinant V antigen, protein A-V antigen fusion peptide, and recombinant protein A to provide passive protection in mice against 10 minimum lethal doses of Lcr^+ cells of *Yersinia pestis* KIM, *Yersinia pseudotuberculosis* PB1, and *Yersinia enterocolitica* WA. Mice were challenged intravenously and γ-globulins were then administered intravenously on postinfection days 1, 3, and 5 at, unless indicated otherwise, a dose of 100 μg. Lengths of solid bars show survival in days of individual mice eventually succumbing to infection; open bars represent survival of independent infected mice until the experiment was terminated at 21 days. Numbers adjacent to solid bars show mean survival time in days.

FIGURE 6. Immunoblots prepared with rabbit anti-native V antigen (A) or mouse monoclonal 17A5.1 anti-V antigen (B) directed against truncated protein A (PA) (lane 1), protein A-V antigen fusion peptide (PAV) (lane 2), PAV partially hydrolyzed by formic acid (lane 3), PAV partially hydrolyzed by formic acid and passed through the IgG Sepharose 6FF column (lane 4), whole Lcr^+ cells of *Yersinia pestis* KIM (lane 5), and whole Lcr^- cells of *Y. pestis* KIM (lane 6); A-V_d, V_o, V_d, and A indicate the positions of PAV, native V antigen (37 kDa), truncated V antigen (29.5 kDa), and truncated Protein A, respectively. Human γ-globulin was used to block nonspecific reactions of monoclonal antibodies against IgG binding domains of Protein A (26).

FIGURE 7. Immunoblots prepared with rabbit anti-protein A-V antigen fusion peptide (A) and anti-truncated protein A (B) directed against whole cells of *Escherichia coli* containing the vector plasmid pKK223-3 (lane 1) or recombinant plasmid pKE14 (lane 2). Also shown are reactions against disrupted and centrifuged whole cells of *E. coli* (pKE14) (lane 3) and further fractionation of V antigen by chromatography on phenyl-Sepharose CL-4B (lane 4), DEAE cellulose (lane 5), Sephacryl S-300SF (lane 6), calcium hydroxylapatite (lane 7), and a second passage on DEAE cellulose (lane 8).

While the forms of the invention herein disclosed constitute presently preferred embodiments, many others are possible. It is not intended herein to mention all of the possible equivalent forms or ramifications of the invention. It is understood that the terms used herein are merely descriptive rather than limiting, and that various changes may be made without departing from the spirit or scope of the invention.

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